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Involvement of PPARα and PPARγ in apoptosis and proliferation of human hepatocarcinoma HepG2 cells

Marina Maggiora, Manuela Oraldi, Giuliana Muzio and Rosa Angela Canuto*

Dipartimento di Medicina ed Oncologia Sperimentale, Università di Torino, Corso Raffaello, Turin, Italy

Peroxisome proliferator-activated receptors (PPARs) mediate the effects of various ligands, known as peroxisome proliferators, a heterogeneous class of compounds including industrial chemicals, pharmaceuticals, and biomolecules such as fatty acids and eicosanoids. Among peroxisome proliferators, fibrate derivatives are considered specific ligands for PPARα, whereas eicosanoids, such as PGJ2, for PPARγ. The study aimed to clarify the relation between PPARs and apoptosis or proliferation on the same type of cells, using clofibrate as specific ligand of PPARα and PGJ2 as specific ligand of PPARγ. The cells used were human hepatocarcinoma HepG2 cells. The results showed that PPARα protein content increased in HepG2 cells treated with clofibrate, causing apoptosis in a time- and concentration-dependent way, as evidenced by the citofluorimetric assay and determination of BAD, myc and protein phosphatase 2A protein content. It also emerged that PPARγ increased in the same cells when treated with a specific ligand of this PPAR; in this case the increase of PPARγ did not cause an increase of apoptosis, but a time- and concentration-dependent inhibition of cell proliferation, evidenced by decreased cell numbers and increased number of cells in the G0/G1 phase of the cycle. It may be concluded that PPARα is chiefly related to apoptosis and PPARγ to cell proliferation. Copyright © 2010 John Wiley & Sons, Ltd.

KEY WORDS — PPARα; PPARγ; HepG2 cells; apoptosis; clofibrate; PGJ2

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) mediate the effects of various ligands, known as peroxisome proliferators, a heterogeneous class of compounds including industrial chemicals, pharmaceuticals, and biomolecules such as fatty acids and eicosanoids. PPARs are a subgroup of the nuclear hormone receptor superfamily, comprising three molecular forms, namely PPARα, β/δ, and γ, that are differentially expressed in adult and embryonic tissues.[1] Upon ligand binding, PPARs dimerize with retinoid receptors, translocate to the nucleus, recognize specific PP-responsive elements (PPRE) on DNA, and transactivate a number of genes.[2,3] PPARs are involved in the regulation of processes such as intracellular lipid trafficking, mitochondrial and peroxisomal fatty acid uptake and β-oxidation, inflammation, cell proliferation, and death. In addition, depending on the environment, it has been proposed that they act either as tumor suppressors or as tumor promoters.[4]

Among peroxisome proliferators, fibrate derivatives are considered specific ligands for PPARα, whereas eicosanoids, such as prostaglandin (PG)J2, are ligands for PPARγ. Fibrate derivatives include agents that have been and still are widely used as hypolipidemic drugs, thanks to their ability to lower plasma triglyceride levels by accelerating mitochondrial fatty acid β-oxidation through PPARα activation.[5] Their administration to rats and mice causes peroxisome proliferation, liver hypertrophy and hyperplasia, and hepatocarcinogenesis;[6,7] thus fibric acid derivatives, such as clofibrate, have been widely employed in hepatocarcinogenesis protocols for rodents,[8–10] in which its antiapoptotic action is assumed to play an importantrole. On the contrary, monkeys, pigs, and humans appear quite resistant to such effects.[11–13] Peroxisome proliferators are usually regarded as non-genotoxic carcinogens, and their oncogenicity seems to depend on the oxidative stress caused by peroxisome proliferation as well as on their ability to alter the balance between cell proliferation and death.[14,15] This action is mediated through activation of PPARα, as shown by studies reporting that PPARα-null mice are refractory to hepatocarcinogenesis induced by administration of long-term peroxisome proliferators.[16]

However, previous results from our laboratory have demonstrated that treatment with clofibrate promptly induces a massive and typical apoptosis in hepatocarcinoma cells of both murine (Yoshida AH-130) and human origin (HepG2).[17] These observations have since been extended to other peroxisome proliferators: nafenopin induces apoptotic death in AH-130 cells,[17] perfluorooctanoic acid and BR931 exert cytotoxic effects on the HepG2 hepatocarcinoma cell line,[18,19] and conjugated linoleic acid induces apoptotic

* Correspondence to: R.A. Canuto, Dipartimento di Medicina ed Oncologia Sperimentale, Università di Torino, Corso Raffaello 30, 10125 Torino, Italy.
Tel: +39 011 6707781; Fax: +39 011 6707753.
E-mail: rosangela.canuto@unito.it

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death or inhibits cell proliferation depending on the degree of malignancy of hepatic tumor cells.[20,21] In addition, WY-14 643 has been shown to enhance hepatocyte apoptosis in mouse liver.[21] Cytotoxicity or cell proliferation inhibition by peroxisome proliferators is not restricted to cells of hepatic origin, since similar effects have been observed in breast and lung cancer cell lines[20,22–25] as well as in human keratinocytes and lymphoblasts.[26,27] The effect of peroxisome proliferators on apoptosis or on cell proliferation may be mediated by activation of PPARs. Ours previous results appear to suggest that induction of PPARα is involved in the effect of peroxisome proliferators on apoptosis, whereas induction of PPARγ is involved in the effect of peroxisome proliferators on cell proliferation inhibition. For example, clofibrate induced apoptosis in human keratinocytes through induction of PPARα and inhibition of HMG-CoA reductase (HMGR), a key enzyme in isoprenoid biosynthesis.[26] Conjugated linoleic acid induced apoptotic death or inhibited cell proliferation in several tumor cell lines, respectively through PPARα or PPARγ activation.[20,21] On the contrary, other studies have found that PPARγ activation triggers apoptosis both *in vitro* and *in vivo.* [28–30]

The present study aimed to clarify the relation between PPARs and apoptosis or proliferation on the same type of cells, using clofibrate as specific ligand of PPARα, and PGJ2 as specific ligand of PPARγ. The cells used were human hepatocarcinoma HepG2 cells.

MATERIALS AND METHODS

Reagents

Antibodies were purchased from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA); MEM, fetal calf serum (FBS) from Cambrex Corporation (East Rutherford, NJ), PGJ2 from Cayman Chemical (Ann Arbor, MI); the other reagents from Sigma (St. Louis, MO).

Cell cultures

Human hepatocarcinoma HepG2 cells (ATCC, Manassas, VA, USA) were seeded (30 000 cells cm^{-2}) and maintained for 24 h in MEM medium supplemented with 2 mM glutamine, 1% antibiotic/antimycotic solution, 1% non-essential amino acids, and 10% FBS at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Clofibrate treatment

Twenty-four hours after cell seeding, clofibrate dissolved in DMSO (maximum final concentration 0.05%) was added at concentrations of 0.25, 0.5, or 1 mM. Control cells were treated with vehicle alone. At the experimental times indicated in the figures, cells treated or not with clofibrate were trypsinized, harvested, and centrifuged at 600*g* for 10 min in order to carry out the determinations described below.

PGJ2 treatment

PGJ2 prepared in methylacetate was evaporated under nitrogen and resuspended in DMSO at 3 mM concentration. Twenty-four hour after cell seeding, PGJ2 was added at concentrations of 5 and 10 μM. Control cells were treated with vehicle alone. At the experimental times indicated in the figures, cells treated or not with PGJ2 were trypsinized, harvested and centrifuged at 600*g* for 10 min in order to carry out the determinations described below.

Cell proliferation

Cell proliferation was measured by counting the number of cells present in the monolayer using a Bürker chamber. The trypan blue exclusion test for viability determination was performed.

DNA content

After treatments, the percentage of cells showing an hypodiploid content of DNA, typical of apoptotic cells (sub G0/G1 peak), was evaluated by determining the DNA content through propidium iodide staining, as described elsewhere.[31] Briefly, cells were washed in PBS, fixed in ice-cold 70% ethanol for at least 30 min, incubated at room temperature in PBS containing DNase-free RNase (Type II-A) and propidium iodide, respectively at final concentrations of 0.4 and 0.18 mg ml^{-1}, then analyzed with a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA) equipped with a 488 nm argon laser and two filters in acquisition, respectively at 585 nm (FL2) and above 620 nm (FL3). Data were recorded on a Macintosh Computer (G3), using CellQuest software (BD Biosciences, Le Pont-De-Claix Cedex France).

Western blot analysis

Collected cells were suspended to 50% (w/v) in HCMF buffer containing 1% Triton, 0.1% SDS, 2 mM $CaCl_2$, 100 μg ml^{-1} phenyl methyl sulfonyl fluoride, 1 μg ml^{-1} leupeptin, and sonicated three times for 3 s. The extracted proteins were used for Western blot analysis, as explained elsewhere.[32] Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratory, Inc., Hercules, CA).

Polyclonal anti-BAD, anti-c-myc, anti-protein phosphatase (PP) 2A, anti-PPARα and γ antibodies, and monoclonal anti-β-actin antibodies were used. Protein bands were visualized through a chemiluminescence detection system (Immun-Star™ HRP Luminol/Enhancer; Bio-Rad Laboratories, Inc.).

Statistical analysis

Data are expressed as means ± SD. The significance of differences between group means was assessed by variance analysis, followed by the *post hoc* Newman–Keuls test.

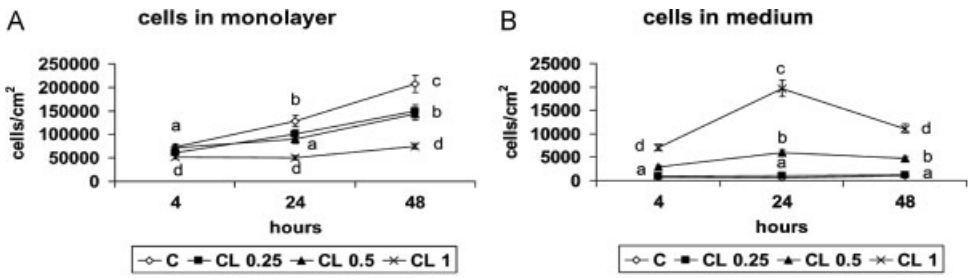

Figure 1. Number of cells present in monolayer and culture medium after treatment of HepG2 cells with clofibrate. Twenty-four hours after seeding, cells were treated with different concentrations (mM) of clofibrate for periods ranging from 4–48 h. Control cells were treated with vehicle alone. The number of cells cm^{-2} counted in the monolayer or in the medium are represented as means ± SD of three experiments. Means with different letters are significantly different from one another ($P < 0.05$) as determined by analysis of variance followed by the *post hoc* Newman–Keuls test. C, control cells; CL 0.25, cells treated with 0.25 mM clofibrate; CL 0.5, cells treated with 0.5 mM clofibrate; CL 1, cells treated with 1 mM clofibrate.

RESULTS AND DISCUSSION

To demonstrate the different effect of PPARα and PPARγ on tumor cells, the same HepG2 cells were treated with two different ligands of PPAR isoforms: clofibrate, which is specific for the induction of PPARα, and PGJ2, which is specific for PPARγ.

Figure 1 summarizes the results regarding the growth reduction determined by clofibrate treatment in HepG2 cells. Note that 48 h clofibrate treatment caused a dose-dependent decrease in cell numbers, corresponding, respectively, to 72, 70, and 36% of the control cells at 0.25, 0.5, and 1 mM concentrations. At the same experimental time, the number of cells floating in the medium was equal to that of control cells for 0.25 mM concentration, and about 5 and 10 times control values for the other two concentrations. To evaluate the possible apoptotic effect of clofibrate, DNA content and some pro-apoptotic proteins were analyzed.

Figure 2 shows the DNA content evaluated by flow cytometric analysis: the percentage of cells in apoptosis increased significantly in the presence of clofibrate concentrations at 24 and 48 h, reaching an increase of about 3.5 times versus controls at 48 h with 0.5 mM concentration.

Only the two lowest concentrations were used to analyze some proteins involved in apoptosis. Figure 3 shows that BAD (panel A), c-myc (panel B), and PP2A (panel C) increased versus controls after 48 h treatment with 0.25 and 0.5 mM. BAD is conventionally considered a marker of apoptosis. The ability of Bad to promote apoptosis depends upon its phosphorylation status; when dephosphorylated,

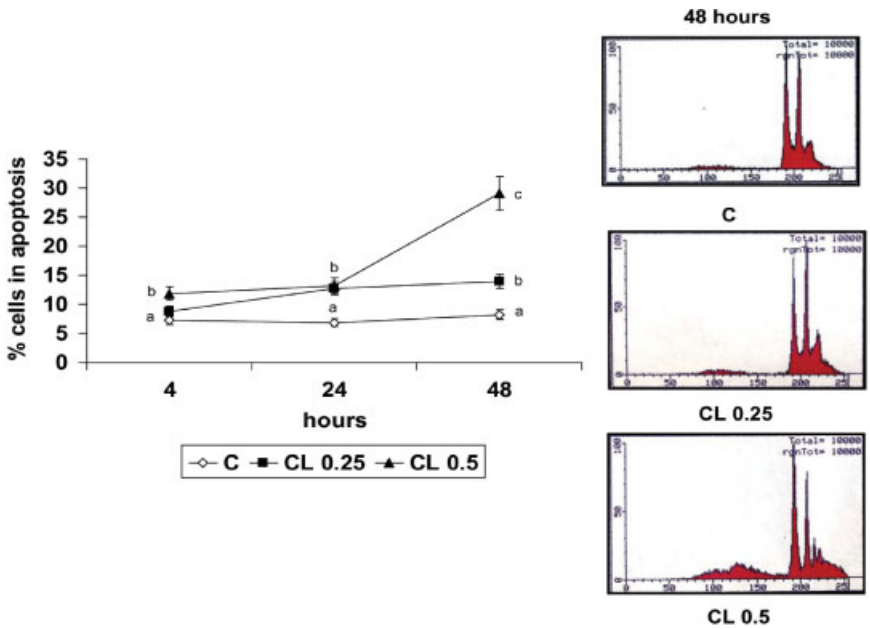

Figure 2. Percentage of apoptotic cells present in the monolayer after treatment of HepG2 cells with clofibrate 24 h after seeding, cells were treated with different concentrations (mM) of clofibrate for periods ranging from 4–48 h. Control cells were treated with vehicle alone. The percentage of apoptotic cells determined by flow cytometric analysis are represented as means ± SD of three experiments. Means with different letters are significantly different from one another ($P < 0.05$), as determined by analysis of variance followed by the *post hoc* Newman–Keuls test. C, control cells; CL 0.25, cells treated with 0.25 mM clofibrate; CL 0.5, cells treated with 0.5 mM clofibrate.

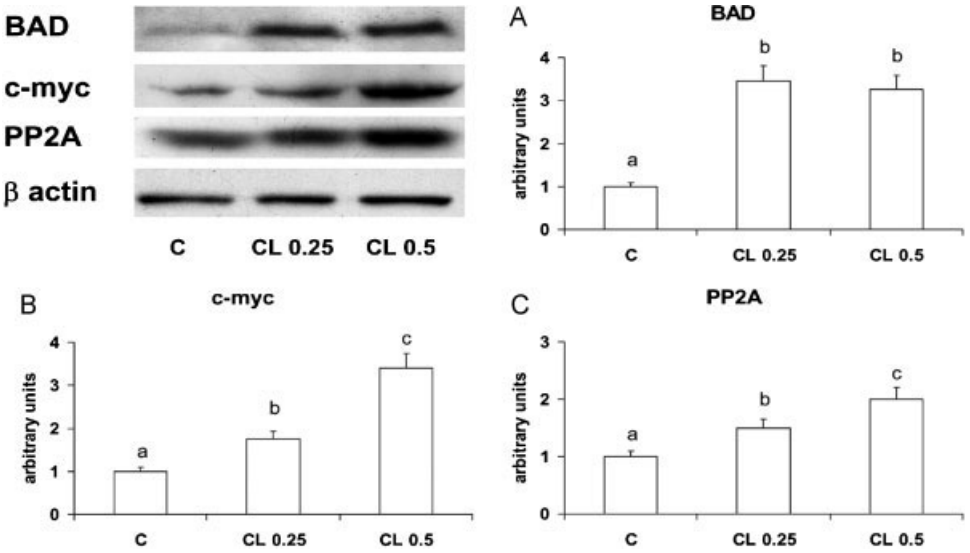

Figure 3. Effect of clofibrate on pro-apototic proteins (BAD, c-myc, PP2A) in HepG2 cells after 48 h of treatment. Protein content was evaluated by Western blot analysis. The densitometry value associated with each protein band was normalized to the corresponding β-actin value and expressed as increasing times, setting the control value (C) arbitrarily at 1. The arbitrary units are means ± SD of three experiments. Means with different letters are significantly different from one another ($P < 0.05$), as determined by analysis of variance followed by the *post hoc* Newman–Keuls test. C, control cells; CL 0.25, cells treated with 0.25 mM clofibrate; CL 0.5, cells treated with 0.5 mM clofibrate.

Bad is targeted to the mitochondria surface where it binds Bcl-X_L or Bcl-2, and causes cell death. Changes in Bad phosphorylation occur rapidly in response to apoptotic stimuli.[33] Therefore, dephosphorylation is an essential stage in apoptosis. The increase of PP2A protein content is probably important in determining Bad dephosphorylation. In fact, PP2A plays a critical role in cellular physiology including cell cycle regulation, cell proliferation and death, development, cytoskeleton dynamics, cell mobility, and regulation of multiple signal transduction pathways.[34] The results of this research agree with our previous data obtained treating hepatocarcinoma SK-HEP-1 with conjugated linoleic acid: in that experimental protocol the induced PPARα increased PP2A causing apoptosis.[21]

The increase in c-myc observed in this research was parallel to the increase in PPARα; this collaborates in sensitizing cells to apoptosis, and agrees with our previous observations that ciprofibrate- or clofibrate-induced apoptosis was coupled with increased content of PPARα and c-myc in hepatoma cells and in keratinocytes, respect-

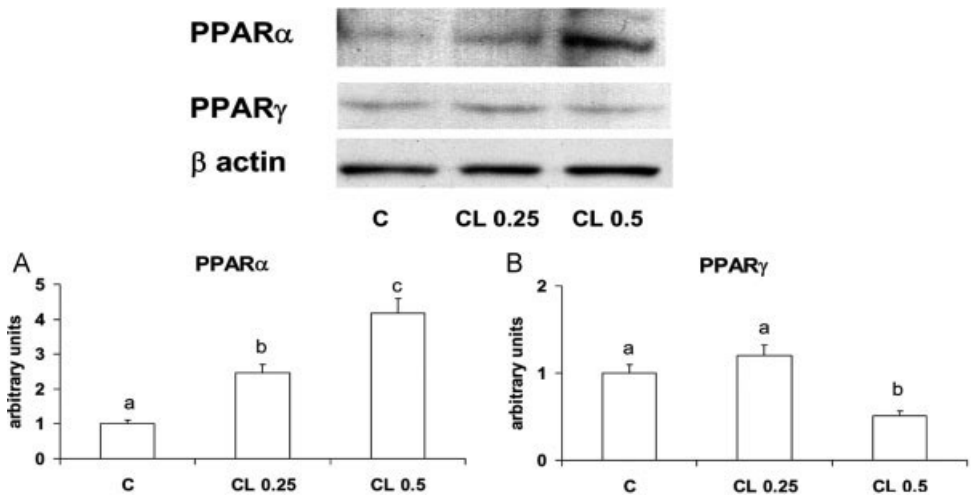

Figure 4. Effect of clofibrate on PPARα and γ protein content in HepG2 cells after 48 h of treatment. Protein content was evaluated by Western blot analysis. The densitometry value associated with each protein band was normalized to the corresponding β-actin value and expressed as increasing times, setting the control value (C) arbitrarily at 1. The arbitrary units are means ± SD of three experiments. Means with different letters are significantly different from one another ($P < 0.05$), as determined by analysis of variance followed by the *post hoc* Newman–Keuls test. C, control cells; CL 0.25, cells treated with 0.25 mM clofibrate; CL 0.5, cells treated with 0.5 mM clofibrate.

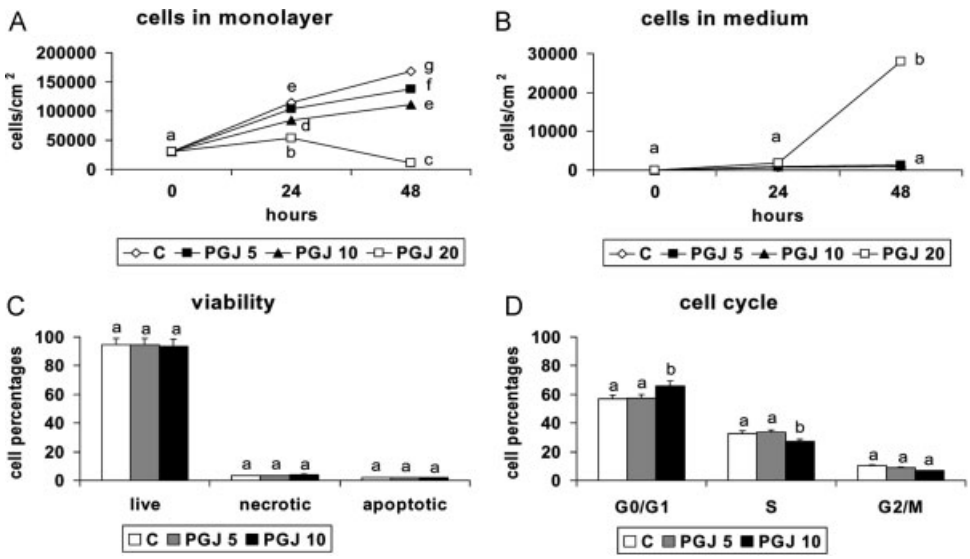

Figure 5. Number of cells in monolayer and medium, percentage of viable, necrotic, and apoptotic cells, and percentage of cells in cell cycle phases, after treatment with PGJ2 of HepG2 cells. Twenty-four hours after seeding, cells were treated with different concentrations (μM) of PGJ2 for a further 24 and 48 h. Control cells were treated with vehicle alone. The number of cells cm^{-2} counted in monolayer or medium, and the percentages of cells in cycle phases, are represented as means ± SD of three experiments. The SD of the percentage of cells in different cycle phases (not reported) was below 10%. Means with different letters are significantly different from one another ($P < 0.05$) as determined by analysis of variance followed by the *post hoc* Newman–Keuls test. C, control cells; PGJ 5, cells treated with 5 μM PGJ2; PGJ 10, cells treated with 10 μM PGJ2; PGJ 20, cells treated with 20 μM PGJ2.

ively.[27,35] The cellular oncogene c-myc encodes a nuclear transcription factor involved in both normal and dysregulated cell division, and/or in the apoptotic process. Some studies[36–38] have shown that the hepatic c-myc (or N-myc) mRNA level is increased in rodents treated with peroxisome proliferators, and that c-myc gene expression is dependent on PPARα, since PPARα null mice were refractory to induction of the c-myc oncogene in liver after treatment with a peroxisome proliferator. Further support for a direct role of PPARα in the regulation of c-myc gene expression has been provided by the observation that the mouse N-myc promoter contains a putative peroxisome proliferator response element (PPRE).[37] c-Myc can collaborate with other proteins to induce apoptosis. In neuroblastoma cells, enforced N-myc expression generally increases the susceptibility of neuroblastoma cells to a variety of death stimuli.[38]

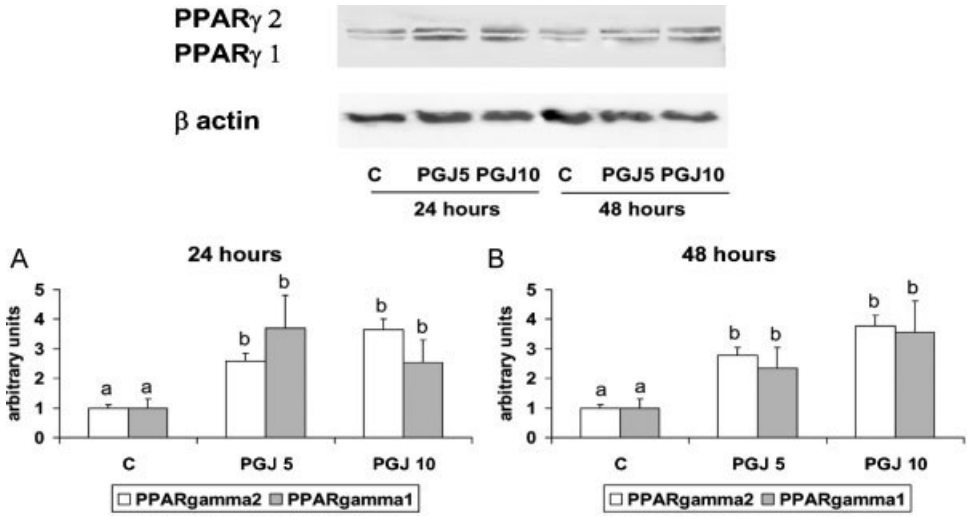

Figure 6. Effect of PGJ2 on PPARγ protein content in HepG2 cells after 24 and 48 h of treatment. Protein content was evaluated by Western blot analysis. The densitometry value associated with each protein band was normalized to the corresponding β-actin value and expressed as increasing times, setting the control value (C) arbitrarily at 1. The arbitrary units are means ± SD of three experiments. For each PPARγ isoform, means with different letters are significantly different from one another ($P < 0.05$), as determined by analysis of variance followed by the *post hoc* Newman–Keuls test. C, control cells PGJ 5; cells treated with 5 μM PGJ2; PGJ 10, cells treated with 10 μM PGJ2.

The evaluation of PPARs showed (Figure 4) that an increase of PPARα (panel A) was evident in the presence of both 0.25 and 0.5 mM clofibrate at 48 h in comparison with controls, whereas PPARγ (panel B) content did not show any increase. This result further confirmed that PPARα is involved in the induction of apoptosis, as hypothesized in the case of our results with clofibrate on keratinocytes, reported elsewhere[26] or with conjugated linoleic acid on hepatoma SK-HEP-1 cells.[21]

To verify the different roles played by PPARα and γ in modulating tumor cell growth, HepG2 cells were treated with PGJ2, a specific natural ligand of PPARγ. 5 or 10 μM PGJ2 decreased cell numbers (Figure 5 panel A and B,), whereas 20 μM PGJ2 caused detachment of almost all cells from the monolayer at 48 h treatment. Therefore, this concentration was not used for the subsequent determinations. In agreement with the fact that no cell detachment was observed in the presence of 5 or 10 μM PGJ2, flow cytometry analysis showed that no changes in necrotic or apoptotic cell numbers had occurred (panel C). The flow cytometric analysis of the percentages of cells in the different cell cycle phases (Figure 5, panel D) revealed that, in the presence of 10 μM PGJ2, the inhibition of cell growth was coupled with a significant increase in the percentage of cells in the G0/G1 phase (+16%), and a significant decrease of S phase. The decreased cell proliferation indicated by the decreased cell numbers and increased cell percentage in the G0/G1 phase, was accompanied by increased PPARγ. Figure 6 shows the PPARγ protein content in PGJ2-treated cells; it may be seen that the increase is time- and concentration-dependent. This confirms other results obtained on different types of cells with other PPARγ-ligands.[11,22,23,30]

CONCLUSIONS

The results of this research confirm that the activation of different PPAR isotypes triggers different intracellular transduction pathways, leading to apoptosis or to the block of cell proliferation. In particular, it emerged that activation of PPARα by clofibrate increases PP2A expression and, in consequence, induces apoptosis by increasing the pro-apoptotic BAD. The importance of this pathway in modulating cell survival had previously been evidenced in hepatoma SK-HEP-1 cells treated with CLA. We also confirm that, in human hepatoma cells, activation of PPARγ preferentially results in a reduction of cell proliferation without inducing cell death. These observations could be important in preparing and using PPAR ligands with the aim of inducing specific effects in tumor cells.

CONFLICT OF INTEREST STATEMENT

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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